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Phytochemicals and antioxidant profile of *Pellacalyx Axillaris*

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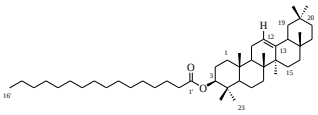
GRAPHICAL ABSTRACT



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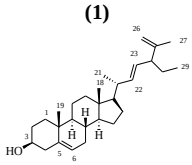

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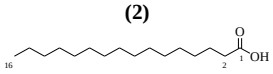

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ABSTRACT
Pellacalyx axillaris (bebuloh) is a mangrove species belongs to Rhizophoraceae family. The ecology of this species takes place across various Southeast Asian countries including Malaysia, Sumatra, Borneo, and the Philippines. In this study, both chemical components and antioxidant activity of different crude extracts from the leaves of *P. axillaris* have been investigated. Dried powdered leaves of *P. axillaris* were extracted through hot technique using three solvents of different polarities; *n*-hexane, ethyl acetate and methanol. Fractionation and purification of *n*-hexane and ethyl acetate extracts via a series of chromatographic techniques yielded a total of three compounds, namely β-amyrin palmitate **(1)**, (24*S*)-ethylcholesta-5,22,25-trien-3β-ol **(2)** and palmitic acid **(3)**. Their structures were identified using spectroscopic methods including Fourier Transfrom Infrared (FTIR), Maas Sspetrometry (MS), ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR). The total phenolic content (TPC) of the extracts was determined using Folin-Ciocalteu assay and their values were in the range of 299 - 532 mg GA (gallic acid)/100 g. The evaluation of antioxidant activity of different crude extracts through the scavenging of free radical DPPH was in the order of methanol (IC_{50} = 34.4 µg/mL) > ethyl acetate (IC_{50} = 89.6 µg/mL) > *n*-hexane (IC_{50} = 858.3 µg/mL). A significantly positive correlation (R^2 = 0.997) was obtained between TPC and the DPPH free radical scavenging at a concentration of 125 µg/mL, for all the crude extracts.

Keywords: P. axillaris,, β-amyrin palmitate, (24S)-ethylcholesta-5,22,25-trien-3β-ol, palmitic acid, Total Phenolic Content (TPC), DPPH free radical scavenging

1. INTRODUCTION

Red mangrove, or known as the Rhizophoraceae family comprised of 120 species over 16 genera, which covered most of tropical and subtropical areas of the world precluding the seasonal regions [1]. The mangrove forests in Malaysia comprised of nine genera with 28 species, which are *Rhizophora*, *Ceriops*, *Bruguiera*, *Pellacalyx*, *Kandelia*, *Anisophyllea*, *Carallia*, *Combretocarpus*, and *Gynotroches* [2]. Most of the species under this family display important roles in human life. For instance, the *Rhizophora mucronata* has been used as construction timber, charcoal and firewood in Kenya [3] while several other Rhizophoraceae species possess great remedial properties, making them good candidates for alternative medicine. For example, the leaves of *B. gymnorhiza* are used to control blood pressure in India [4]. Numerous publications have reported that the chemical constituents of Rhizophoraceae contains both primary and secondary metabolites such as terpenoids, hydrocarbons, sterols, alkaloids, polyphenols and fatty acids [5].

P. axillaris or locally known as 'bebuloh' is a mangrove species that colonizes in damp lowland primary and secondary forests [6]. Its red, hard timber was used to make a durable roof [7]. A detailed taxonamy on this plant has been done by Ding Hou [6] and it was described as a small to medium-sized tree with buttresses, drooping and oblong leaves, growing up to 24 m. Its flowers are small, about 10 mm long, with yellowish green or white petals. Its brown fruits are either oblong or subglobose, about 10 mm by 8 mm in size. Till date, there has been only one paper reported on the isolation of a new tropine alkaloid from the leaves and terminal branches of the species [8]. Therefore, *P. axillaris* was proposed in this study to reveal several other phytochemicals as well as to evaluate the antioxidant property of different crude extracts from the leaves of this species. There have not been many reports on the phytochemistry of genus *Pellacalyx* except for two phytochemical surveys on barks and stems of *P. saccadianus* which have been confirmed to contain alkaloid [9-10]. Recently, the first paper on the isolation of phytochemicals from the leaves of *P. saccadianus* afforded two new constituents, together with six known compounds [11].

2. EXPERIMENTAL

2.1 Plant Materials

P. axillaris samples i.e leaves were collected in July 2014 from Kuala Berang, Terengganu. The species was identified by Dr. Shamsul Khamis from Universiti Putra Malaysia, and a voucher specimen of this species (SK1941/11) was deposited at the Herbarium of Universiti Putra Malaysia.

2.2 Chemicals and Reagents

n-Hexane, ethyl acetate, and methanol were purchased from Sigma Aldrich Company (U.S.A). Sodium carbonate and Folin-Ciocalteu reagent were purchased from Merck (Darmstadt, Germany). 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid (GA) and ascorbic acid (AA) were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). All reagents employed were of analytical grade.

2.3 General Experimental Procedures

Melting points were determined using a hot stage Leica Gallen set up and were not corrected. Functional group analysis was carried out on a Perkin Elmer 1650 FTIR spectrophotometer using Attenuated Total Reflection (ATR) or as KBr pellet. ^{1}H-NMR spectra were recorded on Bruker Avance at 400 MHz and ^{13}C NMR were recorded at 100 MHz, with deuterated chloroform as the solvent. Mass spectral data was acquired by Bruker Mass Spectrometry Services from National University of Singapore (NUS), Singapore. Analytical thin-layer chromatography (TLC) was carried out on Merck precoated SiO2 plate (0.25 mm). Spots were visualized using UV light (254 nm) and sprayed with vanillin reagent. Vacuum liquid chromatography was perfomed using a short column with Merck silica gel 60 (230-400 mesh) while column chromatography (CC) was performed using Merck silica gel 60 (70-230 mesh). The preparative thin-layer chromatography (prep-TLC) was conducted on a thin glass plate coated with Sigma silica gel 60 F_{254} at a thickness of 1.0 mm.

2.4 Extraction and Purification

Powdered leaves (450 g) were extracted in a soxhlet extractor with *n*-hexane (3.5 L) for 18 hr. The solvent was evaporated to dryness using a rotary evaporator to afford an extract PLH (29.95 g, 6.66%). The entire extraction cycle was then performed with ethyl acetate and methanol to yield PLE (6.25 g, 1.39%) and PLM (19.12 g, 4.25%), respectively.

The extract PLH (10.0 g) was purified over silica gel (230-400 mesh) using VLC with a solvent gradient of increasing polarity: *n*-hexane, *n*-hexane: ethyl acetate (49:1, 19:1, 93:7, 9:1, 17:3, 4:1, 3:1, 7:3, 13:7, 3:2, 11:9, 1:1, 9:11, 2:3, 7:13, 3:7, 1:3 1:4, 1:9) and ethyl acetate to give 21 fractions. These fractions were monitored by TLC, and fractions with similar profiles were combined to afford five major fractions (PLHA-PLHE). PLHB (2.14 g) was purified using CC (2.5 x 40 cm) with silica gel (70-230 mesh) and eluted with increasing solvent polarity: *n*-hexane, *n*-hexane: ethyl acetate (19:1, 9:1,17:3,4:1, 3:2, 1:1) and ethyl acetate. Seventy subfractions were collected and examined by TLC. Fractions 10-20 were combined to give a bright yellow residue (1.39 g). The combined fraction (200 mg) was further purified on a preparative TLC plate with *n*-hexane as the solvent system. The silica coating on the thin glass plate was scrapped into 5 bands. Each band was washed with diethyl ether and was filtered through fluted filter papers (Whatman Filter Paper No.1) to give 5 subfractions (PLHAa-PLHAe). All five subfractions were tested using TLC. Fractions PLHAa-PLHAe were combined and concentrated under reduced pressure to give β-amyrin palmitate **(1)**, as a pale yellow solid (12.1 mg). Major fraction PLHD (0.68 g) was subjected to CC (2 x 30 cm) over silica gel eluting sequentially with *n*-hexane, *n*-hexane : ethyl acetate (19:1, 9:1, 4:1, 3:2, 1:1) and ethyl acetate. Fifty fractions were collected and screened by TLC. Subfractions 42-48 exhibited a similar profile with one purple spot on TLC, they were therefore combined and recrystallized from ethyl acetate to yield (24*S*)-ethylcholesta-5,22,25-triene-3β-ol **(2)** as needle-like crystals (5.7 mg, 0.84%).

The ethyl acetate extract, PLE (5.0 g) were fractionated using VLC of silica gel (230-400 mesh) as the stationary phase. The column was first eluted with *n*-hexane, and the polarity was increased gradually using *n*-hexane: ethyl acetate (49:1, 19:1, 93:7, 9:1, 11:4, 17:3, 83:17, 4:1, 3:1, 7:3, 13:7, 3:2, 11:9, 1:1, 2:3, 3:7, 1:4, 1:9) and ethyl acetate. Nineteen fractions were obtained and screened using TLC. Fractions with similar profiles were combined to give five main fractions, PLEA-PLEE. The PLEB fraction (0.68 g) was further chromatographed using CC (2.5 x 30 cm) with silica gel (70-230 mesh) as the stationary phase. The column was eluted sequentially using *n*-hexane, *n*-hexane: ethyl acetate (19:1, 9:1 4:1 3:2) before flushing with ethyl acetate. Thirty-nine fractions were obtained and monitored using TLC. The TLC plate for fraction 24-30 showed two spots, one of which displayed similar R_f value to that of β-amyrin palmitate. The combined fractions (24-30) (100 mg) was further purified using preparative TLC, with *n*-hexane: ethyl acetate (49:1) as the mobile phase. The silica gel coating was later scrapped into 4 bands. Every band was washed using diethyl ether and filtered through fluted filter paper (Whatman No. 1) to afford four fractions (PLEBa-PLEBd). All four fractions were tested on a single TLC plate. Since all four of them showed a similar TLC profile, they were then combined and evaporated to dryness to afford β-amyrin palmitate **(1)** (7.2 mg)

Major fraction PLED was purified over CC using 1.5 x 30 cm column containing 17 g silica gel and *n*-hexane as the mobile phase. The polarity of the solvent was increased gradually using *n*-hexane: ethyl acetate (9:1, 4:1, 3:2, 1:1) and ethyl acetate. Fifty fractions were collected and screened using TLC. Fractions (38-40) showed similar TLC profiles, thus they were combined to give a brown waxy substance which was identified as palmitic acid **(3)** (2.6 mg, 0.63%).

2.4.1 β-amyrin palmitate **(1)**

Pale yellow solid (12.1 mg), m.p. 71-73°C lit. [12] m.p. 64-67°C. TLC: R_f = 0.12 in *n*-hexane. IR (KBr) cm^{-1}; 2921, 1725 and 1656. EIMS: *m/z* 665 (2%) (M^+ + 1) ($C_{46}H_{80}O_2$), 409 (4%), 203 (18%), 218 (100%), ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 0.85 (2 x 3H, s, 3H-24, 3H-28), 0.88 (3H-16'),), 0.89 (4 x 3H, s, 3H-30, 3H-29, 3H-25, 3H-23), 0.89 (1H, H-5), 0.98 (3H, s, H-26), 1.13 (2H, m, H-15), 1.15 (1H, m, H-16a), 1.15 (1H, m, H-7b), 1.25 (1H, m, H-16b), 1.25 (1H, m, H-22b), 1.25 (1H, m, H-7a), 1.26 (10 x 2H, H4'-15'), 1.31 (IH, m, H-1ax), 1.55 (1H, m, H 6b), 1.58 (1H, m, H-6a), 1.60 (1H, m, H-1eq), 1.61 (2H, m, H-3), 1.61 (1H, m, H-9), 1.63 (1H, m, H 11a), 1.63 (1H, m, H-22a), 1.65 (IH, m, H-2a), 1.85 (1H, m, H-2b), 1.95 (1H, m, H-18), 1.95 (1H, m, H-11b), 2.32 (2H, dd, *J* = 7.2, 7.6 Hz, 2H-2), 4.53 (1H, dd, *J* = 8.0, 7.8 Hz, H-3), 5.20 (1H, t, *J* = 3.6 Hz, H-12). ^{13}C-NMR ($CDCl_3$, 100 MHz): 14.2 (C-16'), 15.6 (C-26), 16.7 (C-25), 16.8 (C-24), 18.2 (C-6), 22.7 (C-15'), 23.5 (C-2), 23.6 (C-11), 23.7 (C-30), 25.2 (C-3'), 26.0 (C-27), 26.1 (C-16), 26.9 (C-15), 28.4 (C-23), 28.9 (C-28), 29.1-29.8 (C-4' – C-13'), 31.1 (C-20), 31.9 (C-14'), 32.5 (C-7), 32.6 (C-17), 33.3 (C-29), 34.7 (C-21), 34.9 (C-2'), 36.8 (C-10), 37.1 (C-22), 37.7 (C-4), 38.2 (C-1), 39.8 (C-8), 41.7 (C-14), 46.8 (C-19), 47.2 (C-18), 47.5 (C-9), 55.2 (C-5), 80.6 (C-3), 121.6 (C-12), 145.2 (C-13), 173.8 (C=O).

2.4.2 (24*S*)-ethylcholesta-5,22,25-triene-3β-ol **(2)**

Colorless needles (5.7 mg, 0.84%), m.p. = 143-146°C lit. [13] 141-144°C. TLC: R_f = 0.28 in *n*-hexane: ethyl acetate (19:1). IR νmax (NaCl) cm^{-1}: 3426 (OH), 3085 (=CH), 1640 (C=C), 885 (=CH_2), 2922, 1454, 1380 (CH3). EIMS *m/z* 410 (20%) $[M]^+$ ($C_{29}H_{46}O$), 300 (40%), 273 (40%), 271 (100%), and 255 (60%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.70 (3H, s, H-18), 0.84 (3H, t, *J* = 7.2 Hz, H-29), 1.02 (3H, s, H-19), 1.04 (1H, m, H-15b), 1.08 (1H, m, H-14), 1.09 (1H, m, H-1b), 1.15 (2H, m, H-12), 1.17 (1H, m, H-17), 1.18 (1H, m, H-16b), 1.46 (2H, m, H-28), 1.49 (2H, m, H-7), 1.51 (1H, m, H-11a), 1.53 (1H, m, H-15a), 1.66 (3H, brs, H-27), 1.67 (1H, m, H-16a), 1.86 (1H, m, H-1a), 1.87 (2H, m, H-2), 1.97 (1H, m, H-8), 2.03 (1H, m, H-20), 2.42 (1H, q, *J* = 7.2 Hz, H-24), δ 3.53 (2H, m, H-3), 4.71 (2H, brd, *J* = 1.6 Hz 2H-26), 5.15 (1H, dd, *J* = 7.2, 15.2 Hz, H-23), 5.23 (1H, dd, *J* = 7.2, 15.2 Hz, H-22), 5.36 (2H, d, *J* = 5.2 Hz, H-6); ^{13}C-NMR (100 MHz, $CDCl_3$): δ 12.0 (C-18), 12.1 (C-29), 19.3 (C-19), 20.1 (C-21), 20.7 (C-27), 20.8 (C-11), 24.3 (C-15), 25.7 (C-28), 28.6 (C-16), 31.6 (C-2), 31.8 (C-7 & C-8), 36.5 (C-10), 37.2 (C-1), 39.6 (C-12), 40.1 (C-20), 42.2 (C-4), 42.3 (C-13), 50.1 (C-9), 51.9 (C-24), 55.8 (C-17), 56.8 (C-14), 71.8 (C-3), 109.5 (C-26), 121.5 (C-6), 130.0 (C-23), 137.1 (C-22), 140.8 (C-5), 148.6 (C-25).

2.4.3 Palmitic acid **(3)**

Brown waxy substance (2.6 mg, 0.63%), R_f = 0.31 in *n*-hexane: ethyl acetate (9:1). IR νmax (KBr) cm^{-1}; 3410-2500 (br) (OH), 2917 (C-H), 1700.6 (C=O) cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.90 (3H, t, *J* = 6.4 Hz, H-14), 1.27 (8 × 2H, m, CH2), 1.66 (2H, m, H-3), 2.35 (2H, t, *J* = 7.6 Hz, H-2). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 14.1 (C-14), 22.7-31.9 (8 × CH2), 24.6 (C-3), 33.9 (C-2), 179.4 (C=O).

2.5 Antioxidant Activity

2.5.1 Total Phenolic Content (TPC)

The total phenolic content of crude ethanol extract was evaluated using Folin-Ciocalteu assay adapted to a 96-wells plates as described in Kassim *et. al* [14], with minor modifications. Briefly, 40 µL of samples (125 mg/L) were allowed to react with 20 µL Folin-Ciocalteu's phenol reagent and allowed to stand at room temperature for 5 min. The experiment proceeds with the addition 80 µL sodium carbonate solution (60 g/L) and final treatment with 60 µL distilled water. The mixture was incubated in the dark for 60 minutes, under ambient temperature. After that, the absorbance was recorded using a UV-Vis spectrophotometry, at the wavelength of 760 nm. The TPC of crude extracts (PLH, PLE and PLM) were determined from the calibration curve which was construed using gallic acid (7.18 - 250 mg/L) and the values were expressed in terms of milligram of gallic acid equivalents (GAE) per 100 g of extract.

2.5.2 Antioxidant Activity by DPPH Free Radical Scavenging Assay

The free radical scavenging activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) was carried out on the crude extracts (PLH, PLE, PLM) and the pure compounds, with ascorbic acid as the standard [15]. Stock solutions of extracts (1.0 mg/mL) were diluted with methanol to get to the final concentration range from 15.625-1000 mg/L. The purple colour solution of DPPH was prepared by dissolving DPPH (4 mg) in methanol (100 mL). The prepared sample solutions (100 µL) were allowed to react with the methanolic DPPH solution (100 µL) at room temperature for 30 min. A microplate reader has been used to measure the absorbance of the reaction mixtures at a wavelength of 517 nm. The absorbance of the control (DPPH and

methanol) was measured immediately at 0 min. The percentage of antioxidant activity was calculated using the following equation

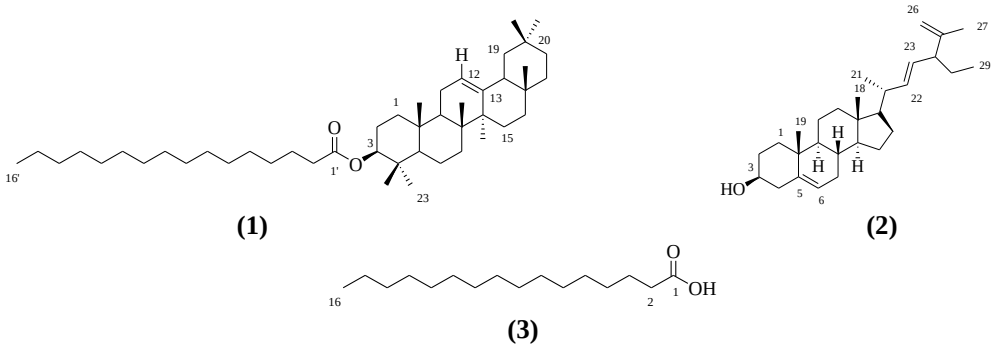
$$\% \text{ Scavenging (I \%)} = \frac{\text{Abs (control)} - \text{Abs (DPPH+sample)}}{\text{Abs (control)}} \text{ x } 100\%$$

By plotting a graph of I% versus concentration of sample or standard, the concentration of sample at 50% inhibition (IC_{50}) can be calculated based on the formula obtained from the plot. All data were run in triplicates and expressed in average value.

3. RESULTS AND DISCUSSION

3.1. Phytochemicals from *P. axillaris*

Fractionation of PLH (10.0 g) using VLC over silica gel afforded five new major fractions, PLHA-PLHE. Purification of PLHB and PLHD yielded β-amyrin palmitate **(1)** and (24S)-ethylcholesta-5,22,25-trien-3β-ol **(2)**. Fractionation of PLE (5.0 g) using VLC with silica gel (230-400 mesh) as the stationary phase, eluted with sequential increasing order of solvent polarity yielded five major fractions, PLEA-PLEE. Further purification of PLEB using CC and preparative-TLC also yielded compound **(1)**. Purification of the major fraction PLED has led to the isolation of a brown waxy substance identified as palmitic acid **(3)** (2.6 mg, 0.63%).



(1) **(2)** **(3)**

3.1.1 β-Amyrin palmitate **(1)**

Continuous elution of the subfraction PLHB yielded a bright yellow residue, which was later chromaographed using preparative-TLC with *n*- hexane as the eluent to give compound **(1)** (12.1 g) as a pale yellow solid, m.p. 71-73°C; lit. [12] m.p. 64-67°C, with R_f value of 0.12 in hexane. The IR spectrum of this compound showed a strong band at 1725 cm^{-1}, corresponding to the stretching vibration of the carbonyl group of an ester, whereas a weak peak at 1656 cm^{-1} indicated the presence of an olefinic bond.

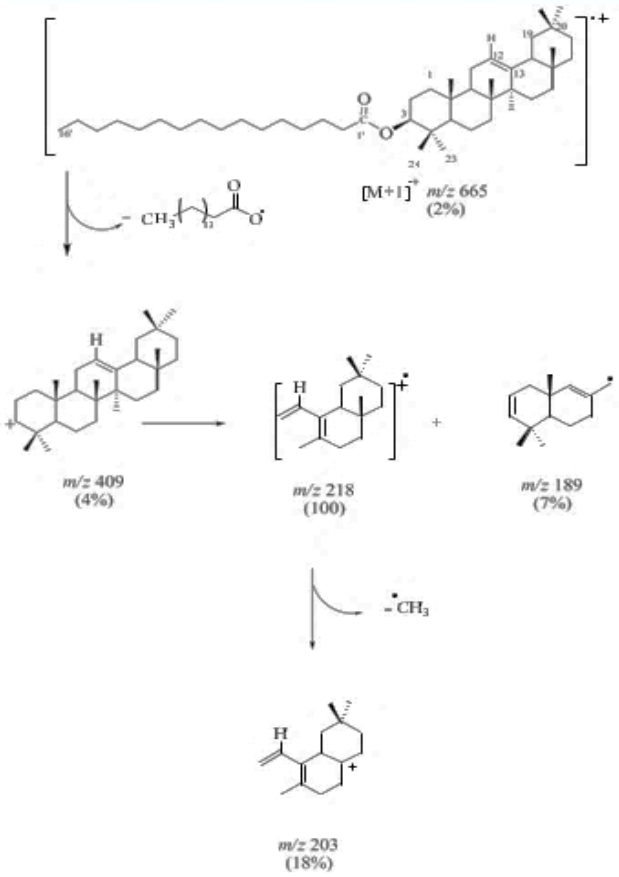
The NMR spectral data of compound **(1)** displayed similar facets to that of an esterified triterpene. The ^{1}H-NMR showed the presence of an oxymethine group at C-3 which resonated as a doublet of doublets at δ 4.53 (J = 8.0, 7.6 Hz). In addition, a trisubstituted HC=C bond was indicated by resonances at δ 5.20 (1H, t, J = 3.6 Hz, H-12). The COSY spectrum showed splitting of the oxymethine proton H-3 at δ 4.53 (dd, J = 8.0, 7.6 Hz) as a result of coupling with its neighbouring proton, H-2. Another correlation between the signal of an olefinic proton, H-12 at δ 5.20 (t, J = 3.6 Hz) with H-11 at δ 1.63 (m) was observed, as well.

The ^{13}C-NMR spectrum revealed the existence of 46 C including twenty-four methyl, nine methylene, five methine and eight quaternary carbons. The tertiary methyl groups produced eight signals which appeared as singlets at δ 0.85 (C-24 and C-28), 0.89 (C-23, C-25, C-29 and C-30), 0.98 (C-26) and 1.15 (C-27). The DEPT 90 spectrum revealed that the triterpene resembled a lot more like ü-amyrin rather than α-amyrin. This is due to the presence of four tertiary C-H bonds that are coherent with the structure of the β-amyrin skeleton as α-amyrin should exhibit six C-H signals. The DEPT 45 showed the presence of seven quaternary carbons in addition to carbonyl of ester group at δ 173.8. The methylene groups on the side chain of the esterified fatty acid were observed as a series of ten signals at δ 29.1-29.7 (10 x CH_2, C-4' – C-13', 31.9 (CH_2, C-14')) and 22.7 (CH_2, C-15'), while the terminal methyl group was observed at δ 14.2.

The EIMS spectrum of compound **(1)** supported the esterified triterpene structure, as a weak signal was observed at *m/z* at 665 (2%) $[M+1]^+$, consistent with the molecular formula $C_{46}H_{80}O_2$ with seven degree of saturation. Fragments at *m/z* 409 (6%) and the base peak at *m/z* 218 (100%) embodied the cyclic triterpene β-amyrin ($C_{30}H_{49}$). The occurance of a stable fragment

at *m/z* 218 (100%) is resulted from the retro Diels-Alder cleavage of ring C **(Scheme 1)**, while the fragment at *m/z* 203 aroused from the subsequent loss of a methyl group.

By comparing the spectroscopic data of this compound with previously reported data, compound **(1)** was identified as a triterpene ester, β-amyrin palmitate, a compound from the stalks of *Celastrus rosthornianus* [12].



Scheme 1: Mass fragmentation of β-amyrin palmitate **(1)**

3.1.2 (24*S*)-Ethylcholesta-5,22-25-trien-3β-ol **(2)**

Purification of major fraction PLHD followed by recrystallization with ethyl acetate yielded compound **(2)** (5.7 mg, 0.84%) as needle-like crystals, m.p. 143-146°C lit. [13], 141-144°C. Analysis of the spectroscopic data of compound **(2)** and comparison with that reported in literature indicated the existence of sterol sekeleton. The IR spectrum showed a broad absorption band at 3426 cm^{-1} which was attributed to the strectching frequency of hydroxyl group. The other absorption bands at ν_{max} 3085, 2922 and 1640 were attributed to germinal methylene (=CH_2), C-H (sp^3) and C=C bond, respectively.

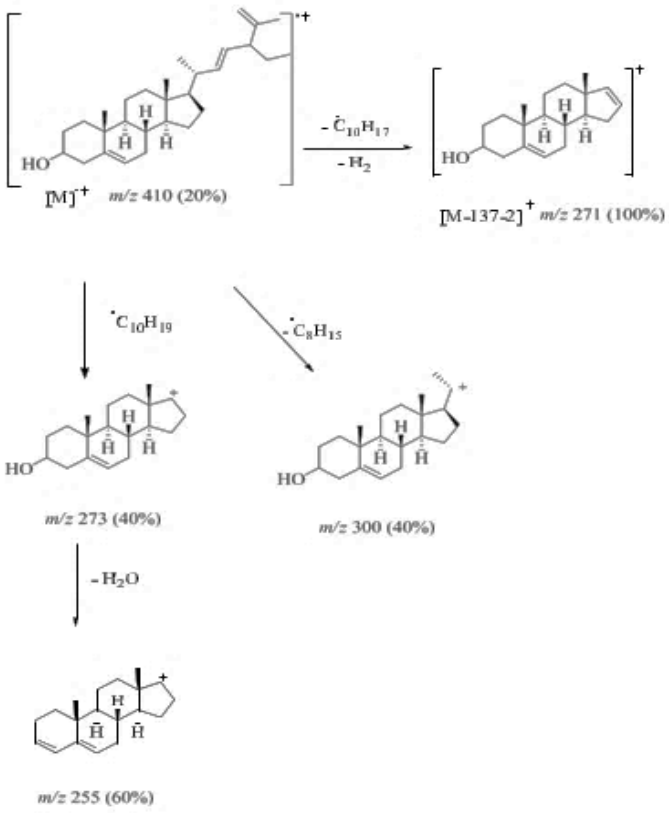
The ^{1}H-NMR spectrum of compound **(2)** resembled to that of phytosterol. In particular, an oxymethine proton appeared as a multiplet signal at δ 3.54 and an olefinic H-6 appeared as a broad doublet signal at δ 5.37 with J = 4.8 Hz. Olefinic protons, at δ 5.23 (1H, dd, J = 7.2, 15.2 Hz) and 5.15 (IH, dd, J = 7.2, 15.2 Hz) were assigned to H-22 and H-23 in trans-orientation. The terminal methylene proton, H-26 was observed as a broad doublet signal with J = 1.6 Hz at δ 4.72 (2H).

The COSY spectrum confirmed the correlation between the signal of an olefinic proton, H-6 at δ 5.37 with that of H-7 at δ 1.49. Two adjacent olefinic methine protons H-22 and H-23 were correlated with one another, and H-23 at δ 5.15 was correlated with the adjacent proton H-24 at δ 2.40 (q, J = 7.6 Hz). An allylic correlation between H-26 and the methyl proton of H-27 was observed in the COSY, confirming that the terminal olefinic proton and methyl group are adjacent to one another.

The ^{13}C-NMR showed 29 peaks, which was in agreement with the presence of twenty-nine carbons in the structure. This finding was supported by the MS, which exhibited a molecular ion peak $[M]^+$ at *m/z* = 410 for $C_{29}H_{46}O$ with seven degree of unsaturation. The DEPT spectrum enables the classification of these 29 carbons into five methyl, ten methylene, ten methine and four quaternary carbons. The resonance at δ 71.8 was assigned to an oxygenated carbon, C-3. Two oleifinic carbons were spotted at δ 140.8 and 121.5 for C-5 and C-6, respectively. Resonances at δ 137.1 and 130.0 were assigned for C-22 and C-23

respectively, while the resonances at δ 148.6 and 109.5 were attributed to the terminal double bonds for quaternary carbon C-25 and methylene C-26, respectively.

The MS spectrum displayed a molecular ion peak $[M]^+$ at *m/z* 410, was consistent with the molecular formula $C_{29}H_{46}O$. Fragments at *m/z* 255, 273, 300, and the base peak at *m/z* 271 resulted from the lost of the side chain, as clearly illustrated in **Scheme 2**. Analysis of the spectroscopic data and comparison study with literature data of a compound from *Solybum mariarum* [13], compound **(2)** was identified as (24*S*)-ethylcholesta-5,22,25-trien-3β-ol.



Scheme 2: Mass fragmentation of (24*S*)-ethylcholesta-5,22,25-trien-3β-ol **(2)**

3.1.3 Palmitic acid **(3)**

Compound **(3)** was obtained as a brown waxy substance (2.6 mg, 0.63%), R_f value of 0.31 in *n*-hexane: ethyl acetate (9:1). Its IR spectrum showed a broad absorption at 3410-2500 cm^{-1} and 1700 cm^{-1} associated with the hydroxyl and carbonyl of a carboxylic acid group, while a strong absorption band at 2917 cm^{-1}, indicated the presence of sp^3 C-H stretching

The ^{1}H-NMR spectrum of compound **(3)** displayed recognizable resonances at δ 2.35 (2H, t, *J* =7.6 Hz, H-2), δ 1.66 (m), that are corresponding to H-3, δ 1.27-1.33 (13 x 2H, m) and at δ 0.90, a triplet *J* = 6.4 Hz attributed to a methyl group. The ^{13}C-NMR spectrum supported the IR spectrum in regard with the presence of a carboxylic group. This spectrum produced a single resonance from the carboxylic acid carbonyl at δ 179.4 (C-1). The molecular ion peak $[M]^+$ was observed in the EIMS spectrum at *m/z* 256, in agreement to a molecular formula of $C_{16}H_{32}O_2$. The EIMS of compound **(3)** and Wiley Library confirmed that the compound was palmitic acid.

Based on the spectroscopic analysis and comparison study with literature data [16] compound **(3)** was identified as palmitic acid or hexadecanoic acid.

3.2 Antioxidant Activity

3.2.1 Total Phenolic Content Determination using the Folin-Ciocalteu (FC) Reagent

The total phenolic content of the three extracts prepared from *P. axillaris* was done using the FC reagent. The results are expressed as equivalents of gallic acid (mg per 100 g extract). As shown in **Table 4**, assays of crude extracts from the

leaves of *P. axillaris* showed that the methanol extract (PLM) had the highest phenolic content (531 mg/100 g), while the n-hexane extract (PLH) had the lowest (299 mg/ 100 g). The total phenolic content of the extracts was increased with increasing solvent polarity, and were in the order of methanol (532 mg/100 g) > ethyl acetate (346 mg/ 100 g) > *n*-hexane (299 mg/ 100 g). Therefore, the use of methanol as the solvent afforded the highest content of phenolic content, which may increase the antioxidant activity of the extract

Table 4: Total phenolic content of different crude extracts from *P. azillaris*

Samples	Total Phenolic Content equivalent to Gallic Acid (mg [2]GAE/100 g)
PLH	299
PLE	346
PLM	532

[1]GAE, R^2= 0.995 at significant value, $p < 0.01$

3.2.2 Free Radical Scavenging using the DPPH Assay

A stable, purple coloured free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH), changes to yellow upon electron transfer (ET) mechanism to an antioxidant. DPPH can react with many samples within a short period of time and is able to detect activities even at very low concentrations [17]. In regard to that, three different crude extracts from *P. axillaris* were screened for their abilities to quench free radicals using DPPH. **Table 5** shows the result of this investigation.

Table 5: IC_{50} values for different crude extracts from *P. axillaris* and standard ascorbic acid.

Samples	DPPH IC_{50} (µg/mL)
PLH	858.53
PLE	89.61
PLM	34.38
AA	25.96

[1]AA = Ascorbic acid

The result revealed that all crude extracts from *P. axillaris* possess antioxidant property. The methanol extract of *P. axillaris*, with an IC_{50} of 34.38 µg/mL showed the highest antioxidant capacity among all extracts tested, however it was still lower that the AA (25.86 µg/mL) standard. The weakest antioxidant capacity was demonstrated by the *n*-hexane extract, followed by the ethyl acetate extract. **Figure 1(a)** shows that the range of inhibition percentage (I%) of the methanolic extract (PLM) from *P. axillaris* is closest to the range of inhibition of the standard compounds AA. This may be due to the presence of the phenolic compounds. Since there was no purification work done to the methanolic extract in this study, therefore the bioactive metabolites that may be responsible for such activity remained unknown.

A simpler way to present the antioxidant activity of different crude extracts from *P. axillaris* is as shown in **Figure 1(b)**. The IC_{50} is the concentration of crude extract required to scavenge 50% of the initial DPPH radicals. Since the relationship between the value of IC_{50} and the percent inhibition is inversely propotional to one another, a lower IC_{50} value correlates to a superior antioxidant activity.

The methanol extracts gave a significant antioxidant activity, compared to the standard ascorbic acid. However, according to the Tukey's multiple comparison tests, there was no significant difference observed between both methanol and ethyl acetate extracts with standard AA. In contrast, a significant difference in antioxidant activity can be seen between the *n*-hexane extract with the the rest of the samples.

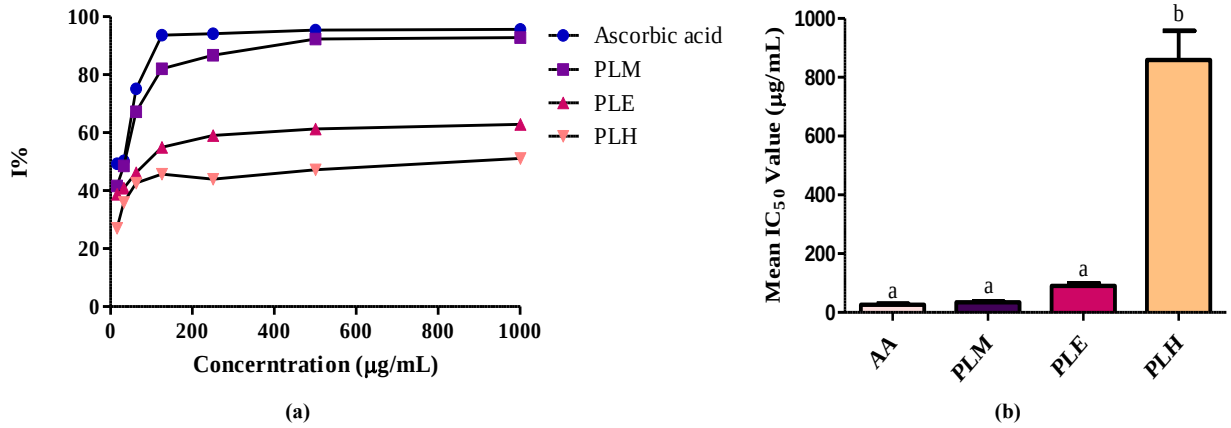


Figure 1: (a) Free radical scavenging against concentration of extracts from leaves of *P. axillaris*, **(b)** Mean IC_{50} value of *P. axillaris* extracts and standard (AA). Different letters are the significant difference measured at $p < 0.05$.

3.2.3 Correlation between Total Phenolic Content (TPC) and Antioxidant Activity

In this study, the relationship of the total phenolic content (TPC) and the inhibition percentage (I%) of the extracts from *P. axillaris* was correlated at a specific concentration of 125 µg/mL using Prism Graphpad, as shown in **Figure 2**. A positive correlation ($R^2 = 0.997$) and a significance level of $p < 0.05$ (one-tailed) were observed, indicating that most of the antioxidant components screened in the total phenolic assay using the FC reagent are phenolics. This suggests that the extracts prepared from the leaves from *P. axillaris*. in this study may consist of phenolic compounds such as flavonoids and tannins. Other studies have also reported that Rhizophoraceae species are rich in tannins [18-19]. In addition, a phytochemical investigation on both stems and twigs of *Rhizophora stylosa* (Rhizophoraceae) yielded a total of seven antioxidant flavonoids [20]. The present work, however, was unable to isolate any bioactive components that might be responsible for this significant antioxidant capacity since the purification work was only done to both *n*-hexane and ethyl acetate extracts.

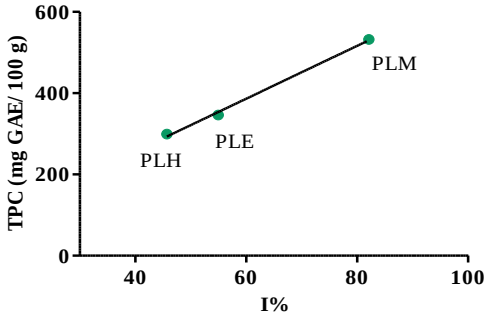


Figure 2: Linear correlation between the total phenolic contents (TPC) and inhibition percentage (I%) of the leaves extract of *P. axillaris* at a concentration of 125 µg/mL.

4. CONCLUSION

Phytochemical analysis of *Pellacalyx axillaris* of Rhizophoraceae revealed the isolation of two classes of natural products which included two triterpenes and one fatty acid. Purification of the *n*-hexane and ethyl acetate extracts of the leaves *P. axillaris* yielded a total of three compounds, β-amyrin palmitate **(1)**, (24S)-ethylcholesta-5,22,25-trien-3β-ol **(2)** and palmitic acid **(3)**. The antioxidant activity screened by two assays, i.e. total phenolic content and free radical scavenging of DPPH revealed that the methanol leaves extract of *P. axillaris* contains the highest amount of phenolic compound, with a TPC value of 532 mg/ 100g and an IC_{50} = 34.38 µg/mL. A positive correlation was observed between total phenolic content and antioxidant value indicating that most of the antioxidant components may be rich in phenolic content. Further recommendations were proposed for future work such as the use of larger quantity of powdered leaves of *P. axillaris* so that more minor compounds can be isolated. The purification of the crude methanolic extract of *P. axillaris* should be considered as well, since the bioactive components which are responsible for its high antioxidant capacity remained unknown. The twigs and stem bark

of this species have not been studied so far, therefore they were suggested for both phytochemical investigation and bioactivity so that a comparison study between different plant parts of *P. axillaris* can be made. Based on the results of antioxidant study of the crude extracts, several other bioactivities including in vitro assays such as antimicrobial, anti-inflammatory and antityrosinase should be conducted, especially to the isolated compounds.

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